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Serum Lipoproteins in Experimental Liver Damage

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LIFE

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CHAPTER I

INTRODUCTION

Alterations in serum lipoproteins in different pathological conditions have been reported(66). These studies have been especially oriented towards the elucidation of atherosclerosis and related syndromes.

For many years the clarity of serum and plasma has suggested that its lipid content is stabilized in solution by linkage to proteins. The properties and structure of the lipoprotein complexes, and the enzyme system responsible for the interaction between lipid and protein are not well known. However, since the synthesis of alpha- and beta-globulins takes place in the liver, it seems of great importance to study the role of the liver in the synthesis of the lipoproteins.

There are a large number of papers devoted to the study of the serum protein fractions of animals with liver damage caused by liver poisons. A review on the effects of carbon tetrachloride was published by Hardin(31), in 1954. Hanada(30), in electrophoretic studies of the plasma proteins in liver impairment found that total protein is normal in rabbits poisoned with carbon tetrachloride and that the alpha-globulin fraction was generally increased. These results are not in agreement with those of Shimura and Kurihara(76) and those of Shirai(77) who reported an increase in alpha- and beta-globulins in this experimental con-

dition.

Usually, carbon tetrachloride is used to induce liver damage, which can be evidenced by histological methods(35)(59)(60). This damage obviously implies all sorts of alterations; e.g. affecting enzymes, such as alkaline phosphatase, succinic dehydrogenase, transaminases(59), etc. Since some of these enzymes are involved in protein synthesis, alterations in serum proteins are to be expected.

Furthermore, alterations in the lipid metabolism have also been observed. Westphal and Priest(87) reported an increase in the electrophoretic mobility of albumin in the serum of rats treated with carbon tetrachloride. The difference in mobility was found to correspond to an increment of approximately one negative charge per albumin molecule. This fact was explained as being due to an interaction of albumin with fatty acids.

In 1951, Koch-Weser, Farber and Popper(35) in biochemical and histological investigations of fatty liver with and without necrosis were able to detect a marked increase in serum phospholipids of rats injected with carbon tetrachloride. Further evidence for the effect of liver damage on lipid transport was furnished by Spitzer and Miller(80) who showed that carbon tetrachloride poisoned dogs transport fat primarily as unesterified fatty acids. It is also well known that cholesterol levels increase under these experimental conditions as shown by Saka et al.(71), among others.

Hartmann, Ruwe and Schulze(32), showed that in dogs poisoned with carbon tetrachloride there is an increase in blood neutral fat and a decrease in phosphatides. Since the transport of fat occurs as neutral fat and cholesterol esters and phospholipids transported in two groups of lipoproteins, the importance of the lipoprotein levels in serum becomes evident.

Little is known about the changes in serum lipoproteins in experimental liver damage by carbon tetrachloride. The most important contribution in this field seems to be that of Pierce and Gofman(62). They studied the effect of carbon tetrachloride poisoning on serum lipoproteins associated with atherosclerosis using ultracentrifugation. They noted that carbon tetrachloride produced an increase of the S_f 3-12 class of lipoprotein in rabbits.

Another aspect of the problem is the effect of carbon tetrachloride on enzymes which are, in a way, related to the lipids of serum and liver. It has been shown that some enzymes, like xanthine oxidase, are activated by carbon tetrachloride(1). In other publications(53)(54) we were able to demonstrate by means of paper electrophoresis that xanthine dehydrogenase activity has the same distribution as the lipoproteins in rat serum as well as in rat liver. Since this enzyme activity is confined to the lipoproteins, it was suggested that the activation by carbon tetrachloride could be attributed to the splitting of the linkages between the enzyme and the lipids.

The explanation given seems to be reasonable since the same fact is observed when milk(63) and liver(85) xanthine dehydrogenase are treated by other organic solvents. Furthermore, it has been well established that xanthine dehydrogenase is obtained from the soluble cytoplasmic fraction of liver homogenates (86), possible exclusive site of fatty acid synthesis(40). Since xanthine dehydrogenase may possibly be loosely bound to lipoprotein or fat bodies, the study of these in serum seems of importance. This binding of xanthine dehydrogenase to lipoproteins at the surface of mitochondria would permit the chain oxidation of xanthine to allantoin to take place within an efficient spatial relationship, once uricase is located in the liver mitochondria.

In view of all these facts, there appears to be a real need for a more detailed study of the effect of carbon tetrachloride on the metabolism of lipoproteins in the liver and, consequentely, to find such variations as are reflected by the blood serum levels of lipoproteins.

Electrophoresis offers the possibility of the easy and inexpensive determination of serum lipoproteins. A great number of supporting media may be used for this purpose, including: starch blocks(8)(37)(38), starch gels(28), agar gels(89), cellulose acetate membranes(36), starch columns(16)(17), etc.

For our study, paper electrophoresis was chosen because of its simplicity and also because it requires only small amounts of serum for the determinations. These advantages over other

techniques are evident. However, some restrictions can be made to most of the procedures so far described for lipoprotein determinations by paper electrophoresis. Among these, inadequate staining of lipoproteins and retention of the dye by the paper itself, stands out. Also the use of rinsing solutions to remove the dye from the paper background may also remove some of the dye from the lipoprotein zones.

Several methods have been proposed for the determination of serum lipoproteins by paper electrophoresis. Among the dyes which have been tried for staining the lipid zones on the paper we can mention: Sudan IV(69)(70)(79), Sudan III(26)(27)(37), Oil Red O(12)(24)(33) and Sudan Black B(67)(82), first introduced in paper electrophoresis by Swahn(81). In our own experience and that of others(66) Sudan Black B seems to be the most suitable dye for lipoprotein staining in paper electrophoresis.

Besides the above mentioned dyes, others have been tried as well. Thus, Loeffler and Wunderly(43) tried a combination of Ciba blue and Sudan Black B and, noir au gras was utilized by Baudoin et al.(5)(6). Common et al.(20) localized lipids by exposing the paper strips to vapors of a 1% osmic acid in 1% chromic acid solution. Recently, Barrollier(4) proposed an interesting method which permits the presentation of double bonds in fatty acids and lipoids, based on a reaction with $AgNO_3$ and iodine bromide and finally dying with Sudan VII B(2)(3).

Some additional procedures have been tried with greater

or less success. Salido(72) included in his studies experiments with osmic acid, dimethylaminoazobenzol, diluted Fuschin, Scarlet R, etc. Searcy et al.(74) proposed a rapid staining of serum lipoproteins and later(73) described the fluorescent detection of these by staining with an acid solution of protoporphyrin.

Some authors have tried to determine serum lipoproteins indirectly by their cholesterol content(13)(39)(56). Nury and Smith(57) proposed a time-saving simplification of the method of Langan, Durrum and Jencks(39) which seems to yield very good results. The most recent aproaches in this respect are those of Crawford(22) and of Searcy et al.(75). The phospholipid content of the lipid zones may also be determined(16)(17)(27)(38). Although these techniques may be used successfully, most authors prefer to use fat-soluble dyes for the study of serum lipoproteins.

It is obvious from an inspection of the literature that Oil Red O and Sudan Black B have been the most widely used stains. Talluto et al.(83) proposed the use of a 50% aqueous solution of diacetin saturated with Oil Red O as a more sensitive method for the quantitative measure of serum lipide fractions. However, the problems already mentioned which are involved in post-staining methods were not solved. The same comments can be extended to the method of Moinat, Appel and Tuller(55) in which the authors proposed the use of a saturated solution of Sudan Black B in a mixture containing 30% methanol, 30% isopropanol, and 40% distilled water.

Casselman(18)(19) in 1954, proposed the use of acetylated Sudan Black B as a more specific histochemical reagent for lipides. From our observations and that of others this reagent is indeed advantageous but still left something to be desired.

In order to avoid the inconvenience so far mentioned, McDonald and Bermes(46)(48), in 1955, proposed a pre-staining method for the determination of lipoproteins by paper electrophoresis. By this new procedure, the lipoproteins are pre-stained (by means of a saturated solution of acetylated Sudan Black B in 95% ethanol) directly in the blood serum sample before application to the paper. The excess of alcohol is evaporated and after the electrophoretic separation the lipoprotein zones appear as blue zones against a practically white background. This technique eliminates the restrictions to the post-staining methods especialy because it avoids washing of the strips and because it gives an almost white background, between the separated zones(49).

The acceptance of this method grew rapidly and a little later Bermes et al.(7) applied the procedure for the determination of the serum lipoprotein levels in rheumatic fever. Bernsohn(8), in 1957, used this method with success in studies on the isolation of serum lipoproteins by zone electrophoresis, and Boggs et al. (11) in lipoprotein studies in cases of idiopathic hyperlipemia. However, the use of ethanol is said to present some possible limitations to the use of this method(78).

In order to avoid this drawback, Solinas et al.(78) in

1957, proposed a method in which diacetin is used as the solvent for Sudan Black B. Nevertheless, Wilcox et al.(88), in 1958, returned to the use of alcohol. They proposed a technique in which the evaporation of the excess alcohol is avoided and claimed very good results and reproducible separations with this new procedure.

Kanabrocki et al.(34) published a modification of the method of McDonald and Bermes(48) which is simple to perform and relatively inexpensive. Later, Mohatgi and Banerjee(68) used the previously described method of McDonald and Bermes(48) in studies of the serum lipoproteins in aminopterin-treated rats. In 1959, Bigler et al.(9) again used the pre-staining method successfully for the study of inborn errors of lipid metabolism.

Among the most recent variations of the pre-staining technique we can mention the one proposed by Larkey and Belko(41) in which a mixture of petroleum ether - ethyl alcohol in the ratio of 1:4 is used as solvent for Sudan Black B.

In our experience we have found that diacetin, although being a good solvent for Sudan Black B, has the inconvenience of being difficult to handle, especially because of its high viscosity. In an attempt to overcome this difficulty, solvents other than ethyl alcohol and diacetin were re-investigated by McDonald and Ribeiro(51). After several trials it was found that both propylene and ethylene glycol possessed some inherent advantages as compared to the solvents previously used.

Later, it was shown that other solvent systems may be

used. Zakelj and Gros(90) proposed the use of a mixture of dioxane and ethylene glycol, while McDonald, Banaszak and Kissane (47)(50) used a system containing ethyl acetate and propylene glycol.

Due to the interest shown by clinical chemists in these pre-staining methods we decided to undertake a more detailed study of the use of propylene and ethylene glycol as solvents for Sudan Black B in the pre-staining of lipoproteins for paper electrophoresis. In this way, we hope to be able to establish a more sensitive and reproducible method and to apply it to the determination of serum lipoprotein levels in experimental liver damage produced by carbon tetrachloride.

CHAPTER II

GENERAL METHODS AND MATERIALS

1. Animals.

Male Wistar white rats weighing between 100 to 150 g were used in the experiments. The animals were maintained on a well balanced standard diet throughout the experiments.

The animals were divided into two groups. One of the groups was kept as controls and the animals from the other group were injected subcutaneously with a single dose of 0.1 ml of carbon tetrachloride per 100 g body weight. It has been shown that carbon tetrachloride is quite effective in producing liver damage at this dosage(1).

2. Blood samples.

Blood samples were withdrawn from both groups of animals by heart puncture under light ether anesthesia. From the injected animals, samples were usually obtained 24 hours after the injection, unless otherwise specified.

The blood was allowed to clot at room temperature and after coagulation it was centrifuged at 2,500 r.p.m., and the clear serum separated. Hemolyzed samples were discarded since it was observed that hemolysis interfered with both protein and lipoprotein determinations by paper electrophoresis. The blood serum

samples were stored at -5ºC for no longer than 3 days, since aging of serum changes the electrophoretic pattern.

3. Staining dyes.

a. Protein staining dye.

Proteins were stained with bromophenol blue obtained from E. Merck Darmstadt, Germany. The dye solutions were prepared as follows:

bromophenol blue ..... 0.1 g
zinc sulfate ..... 50 g
glacial acetic acid ..... 50 ml
made up to 1,000 ml with distilled water.

The procedure used for staining was essentially as follows:

"After the electrophoretic run the strips were removed from the apparatus and dried in an oven at 110-120ºC for 30 minutes in a perfect horizontal position. They were then transfered to the dyeing rack and kept in the dye bath for 16 hours. The strips were then rinsed twice in a 2% (v/v) acetic acid solution for 10 minutes each time, and a third time for 20 minutes. Following this procedure they were placed for 2 minutes in a 2% (w/v) sodium acetate solution containing 10% (v/v) glacial acetic acid. After removing the strips from

the washing bath, they were blotted and dried in a horizontal position for 20 minutes at 120°C. The strips were in this way made ready for color measurements".

b. Lipid dye.

Sudan Black B was the dye selected for lipid staining. Two dye samples were tested; the first was from Hartman-Leddon Company, Philadelphia, Pa., certification No. CZb-5, and the second was obtained from a special experimental lot B.6.56 from the Banting and Best Department of Medical Research of the University of Toronto, Canada, and kindly supplied by Dr. W.G.Bruce Casselman.

Preliminary experiments showed that both samples gave practically the same results and therefore the work was carried out with the commercial sample obtained from Hartman-Leddon Company.

Acetylated Sudan Black B samples had been prepared from the dye lot mentioned above. The procedure used for acetylation was the one suggested by Casselman(42) and essentially it reads as follows:

"Dissolve 1 g of Sudan Black B in 100 ml of diethyl ether and filter the solution in order to remove the insoluble fraction. Under a reflux condenser, heat the ether solution to boiling and add 0.5 ml of acetic anhydride dissolved in 20 ml of ether. Reflux the

mixture for 20 minutes, cool and filter it. Transfer the filtrate to a separating funnel and extract it repeatedly with cold distilled water until the aqueous layer is no longer colored and is not appreciably acidic to "universal" indicator paper. Pour the solution of acetylated Sudan Black B into a dish and evaporate off the ether".

The black product thus prepared had a metallic lustre.

Concentrated solutions of both Sudan Black B and acetylated Sudan Black B were prepared according to the following procedure:

"100 ml of propylene glycol (Eastman Kodak Company, Rochester, N.Y.) are heated to 100-110ºC and 1 g of Sudan Black B (or of the acetylated derivative)is added to the hot solvent, with thorough stirring for 5 minutes. The solution is filtered hot on Whatman No. 2 filter paper, cooled and re-filtered on the same kind of paper.

The concentration of the final solution thus prepared was approximately 0.5 g% with respect to the dye. Care must be taken not to exceed 110ºC when preparing these solutions or else a useless gelatinous mixture will result, as stated in a previous report(51).

4. Paper electrophoresis.

a. Apparatus.

All electrophoretic separations were carried out employing a horizontal strip type of apparatus from Laboratory Glass and Instruments Corp., New York City.

b. Filter paper.

Two kinds of paper were used in preliminary experiments: Whatmann 3MM, and Macherey & Nagel No. 2214 ff, a fat-free type of filter paper.

In both cases the paper strips were 2.5 cm in width and 33 cm long, with an effective length of 28 cm, for potential gradient calculations.

c. Buffer solution.

All electrophoretic separations were carried out in veronal buffer having a pH of 8.6 and an ionic strength of 0.05. This buffer was prepared in the following way:

Stock solution: This was prepared by dissolving 20.60 g of sodium veronal and 3.68 g of veronal in CO_2- free distilled water, the final volume being made up to 1,000 ml.

Working solution: This was obtained by diluting the stock solution to one half its concentration with CO_2-free distilled water.

d. Electrophoretic separations.

The procedure used for the electrophoretic separations was essentially as follows:

"The paper strips are wetted with some of the buffer solution and the excess liquid is removed by blotting. The wetted strips are then placed in the plastic frame of the instrument and adjusted until taut. The strip-holder is then placed in the electrophoretic chamber and the ends of the filter paper strips are dipped into the buffer vessels, containing about 400 ml of buffer solution each. After the frame is in the proper place, the chamber is closed and the power supply turned on (100 volts) for 2 hours in order to achieve equilibrium. After this period of time, the frame was removed and the strips adjusted again so that they were in a perfect horizontal position. The frame was then replaced in the chamber for another 45 minutes with the current on, as before. In all cases the migrant was added after the equilibration period as a thin streak across the width of the strips midway between the ends. After completion of the run the strips were removed and dried always in a perfect horizontal position".

5. Scanning.

The dyed strips for both protein and lipoprotein were scanned with the aid of the Photovolt Transmission Densitometer

Model 525 and the measurements made at 595 mμ. (Photovolt Corporation, New York City).

The instrument was equipped with a scanning stage for semi-automatic plotting or with a Varicord (obtained from the same Company), a variable-response recorder for automatic drawing of the curves.

In the case of protein determinations the strips were clarified with mineral oil prior to scanning.

6. Quantitation of diagrams.

Quantitative determinations were made by planimetry of the areas under the peaks of the electrophoretic curves. The areas were always fixed by extending the curves to the baseline rather than simply dropping perpendiculars downwards from the midpoints of the valleys of the diagrams, when determining proteins. This procedure was followed since it has been shown that drawing perpendiculars may lead to errors when planimetry is carried out(45).

In the case of lipoprotein determination, perpendiculars could be dropped since the error was negligible. Fig. 1 shows an example where the same curve was analyzed by the two processes.

The planimetry of the areas (three determinations in each case) showed the following values (in cm^2), expressed as means plus or minus standard deviations:

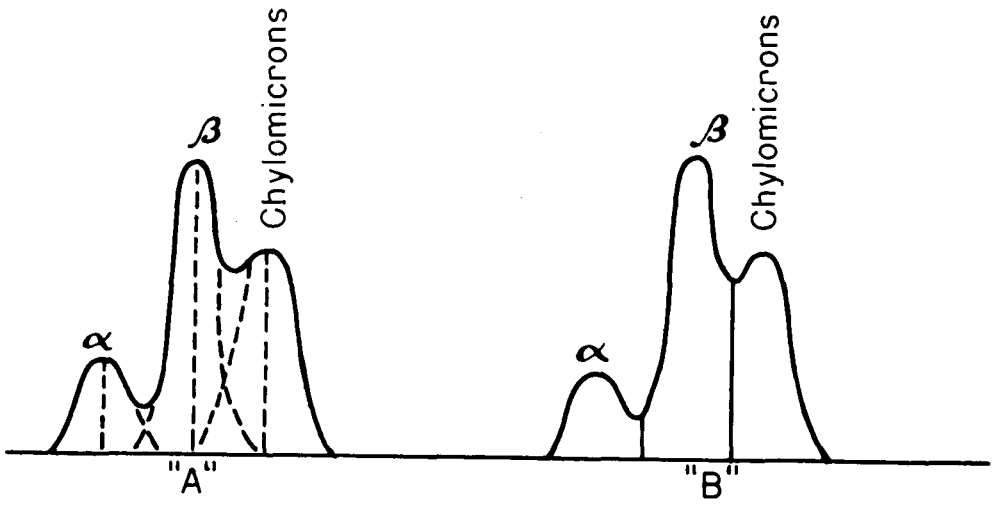

Fig. 1. Quantitation of diagrams by: "A", extending the curves and "B", dropping perpendiculars to base line.

For "A"; alpha = 1.13 ± 0.11, beta = 3.00 ± 0.14, and chylomicrons = 2.50 ± 0.10

For "B"; alpha = 1.10 ± 0.00, beta = 3.06 ± 0.06, and chylomicrons = 2.44 ± 0.08

As can be noted, there is practically no difference between the values of the corresponding fractions and the statistical analysis (student's "t" test) confirmed that no statistical differences could be found between the two sets of measurements (alpha: $t = 0.4762$, $P > 0.6$; beta: $t = 0.7500$, $P > 0.5$; chylomicrons: $t = 0.8571$, $P > 0.4$). This was due to the fact that the valleys of the diagrams are near the baseline, due to the low background in the stained strips.

These are general methods used. In some cases small modifications were introduced and whenever this was done it is so stated in the text. Therefore, unless otherwise specified, the above described methods were employed.

CHAPTER III

PRE-STAINING LIPOPROTEINS FOR ELECTROPHORESIS

As already mentioned in the introductory chapter, in our experience we have found that both propylene and ethylene glycol possessed some inherent advantages as compared to solvents previously used to prepare Sudan Black B solutions. This fact lead us to a more detailed study of the use of propylene and ethylene glycol as solvents for Sudan Black B in the pre-staining of lipoproteins for paper electrophoresis.

1. Preliminary experiments.

Preliminary experiments have shown that propylene glycol solutions of Sudan Black B always contained more dissolved dye than those prepared with ethylene glycol. This can be seen easily by spectrophotometric measurements. The shape of the absorption curves was the same but the propylene glycol solution always presented a greater optical density at practically all wave lengths (from 400 to 700 mμ). This is a clear indication that there is a greater solubility of the dye in propylene glycol. Therefore, this solvent was used in all subsequent experiments.

In an attempt to select, at as early a date as possible, the particular dye to be used in this investigation, Sudan Black B and acetylated Sudan Black B solutions were used in these

preliminary experiments. Using the technique previously described by MacDonald and Ribeiro(51), we were able to show that the acetylated derivative always gave sharper differentiation between the zones of the alpha- and beta-lipoproteins. The results of these experiments are illustrated in Figure 2.

In view of these results and that of others(50)(45) we decided to use acetylated Sudan Black B in our investigation.

Once these two preliminary conditions were established, a detailed study of the conditions for pre-staining lipoproteins was undertaken. The principal aspects of the technique which had to be studied were: (a) the amount of dye solution to be added to the serum; (b) the incubation time of the serum with the dye solution; (c) the amount of pre-stained serum to be applied to the paper strip; (d) the stability of the color of the lipid zones after the electrophoretic separation; (e) the reproducibility and error of the method; (f) the proposed technique.

Naturally, the conditions for the electrophoretic separation itself will also be established in the course of experiments. By trying different sets of conditions it was concluded that, in general, a potential gradient of 8 volt/cm for two hours gave very good results. Nevertheless, the conditions used for any particular type of experiment will always be stated in the course of the explanation.

All determinations were carried out with six different serum samples. However, for simplicity, only one example will be

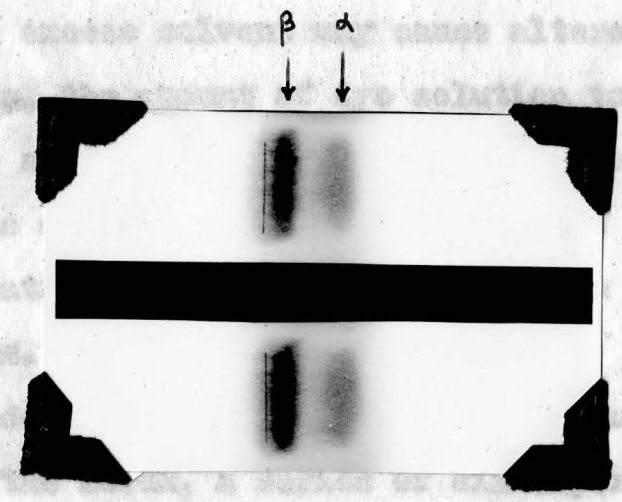

Fig. 2 - Comparison between two runs of the same serum sample: (A) pre-stained with Sudan Black B; (B) pre-stained with acetylated Sudan Black B.

given in each case, the one given being the most representative of the property under study.

2. Amount of dye solution to be added to the serum.

Since excess solvent may cause alterations in the lipoprotein patterns, the amount of dye solution to be added to the serum should be such as to avoid these complications. On the other hand, this amount of dye solution should be sufficiently great to give satisfactory colored lipoprotein zones in the electropherogram.

In order to establish the proper amount of dye solution to be added to the serum, a series of six experiments were undertaken. These consisted of preparing a set of centrifuge tubes, each containing 0.5 ml of fresh human blood serum; to each tube was added slowly, with constant, gentle stirring, increasing amounts of a saturated solution of acetylated Sudan Black B, prepared as previously described. In Table I, is shown the way in which the set of tubes were prepared.

The serum with the added dye solution was left to stand at room temperature for 45 minutes and then centrifuged in an International Clinical Centrifuge Model CL at maximum speed for 15 minutes. The supernatant solution was used for the electrophoretic separations.

Paper electrophoresis was carried out using the procedure described under general methods. Six strips of Whatman 3MM

TABLE I

AMOUNT OF SATURATED SUDAN BLACK B SOLUTION IN PROPYLENE GLYCOL ADDED TO THE SAME SERUM SAMPLE

Tube No.	Amount of serum	Amount of dye soln.
1	0.50 ml	0.025 ml
2	0.50	0.050
3	0.50	0.075
4	0.50	0.100
5	0.50	0.125
6	0.50	0.150

filter paper were mounted in the strip-holder of the instrument and after the equilibration period was over, 20 µl of each of the prepared samples were applied to individual paper strips. The separations were carried out at 25°C, using a potential gradient of 8 volt/cm, and lasted two hours. After this period of time the α-lipoprotein zone had moved about 3 cm from the starting point and the β-lipoprotein fraction moved about 1.2 cm. There is also a lipid zone extending from the origin to the beta-lipoprotein fraction which corresponds to the chylomicrons. This fraction appears as a result of the adsorption of the fat particles having a diameter not lower then 0.5 µ and we have selected blood samples containing more of these than usual, in order to see the effect of it on the beta-lipoprotein fraction. It seems from the diagrams that will follow that this fraction can be determined quite satisfactorily and that the separation between chylomicrons and beta-lipoproteins can be easily ascertained by this method.

After the electrophoretic separations the strips were removed from the instrument chamber and dried in the dark, at room temperature, overnight. Figure 3 shows a typical experiment of this kind.

The dried strips were than scanned in order to evaluate the amount of dye present in each lipid zone. The criteria adopted for selecting the best conditions in this case were to consider the intensity of the background and whether the color of

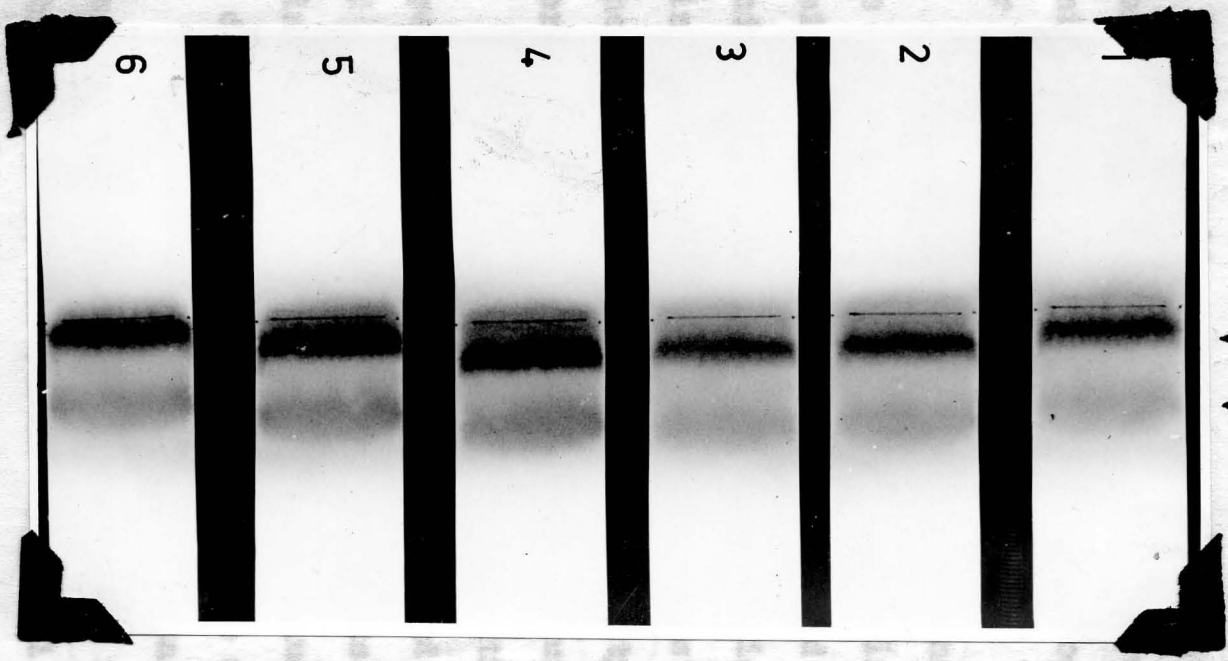

Fig. 3. Paper electrophoresis of the same sample of human blood serum lipoproteins pre-stained with increasing amounts of dye solution. Number in the strips corresponds to the tube number in Table I.

the lipid fractions was measurable within a good range of sensitivity of the scanner.

To avoid an unnecessary excess of illustrations, we are presenting in Figure 4 only the patterns corresponding to the scanning of the strips of serum samples pre-stained as described under Nos. 1, 2, and 3 in Table I.

An analysis of these patterns clearly indicates that there are increased amounts of color in the lipid zones with increasing amounts of dye solution added to the serum. The background is quite low and therefore satisfactory for the purpose of the investigation. The remaining question would be to find out whether the increase in color intensity of the lipid zones was proportional to the amount of dye solution added to the serum.

In order to verify the relationship between the amount of dye solution added to the serum and the color intensity of the lipid zones, the areas under the peaks in the diagrams were determined by planimetry. The mean values obtained, after duplicate determinations of each area, are shown in Table II.

As can be seen from the data in Table II, there is not a constant increase of the areas with increasing amounts of dye solution added to the serum. The real dependence between amount of dye and color intensity of the lipid zones can better be evaluated when the areas are plotted on a graph against the amount of dye solution present in the aliquot applied to the paper strip. A typical experiment of this kind is shown in Figure 5.

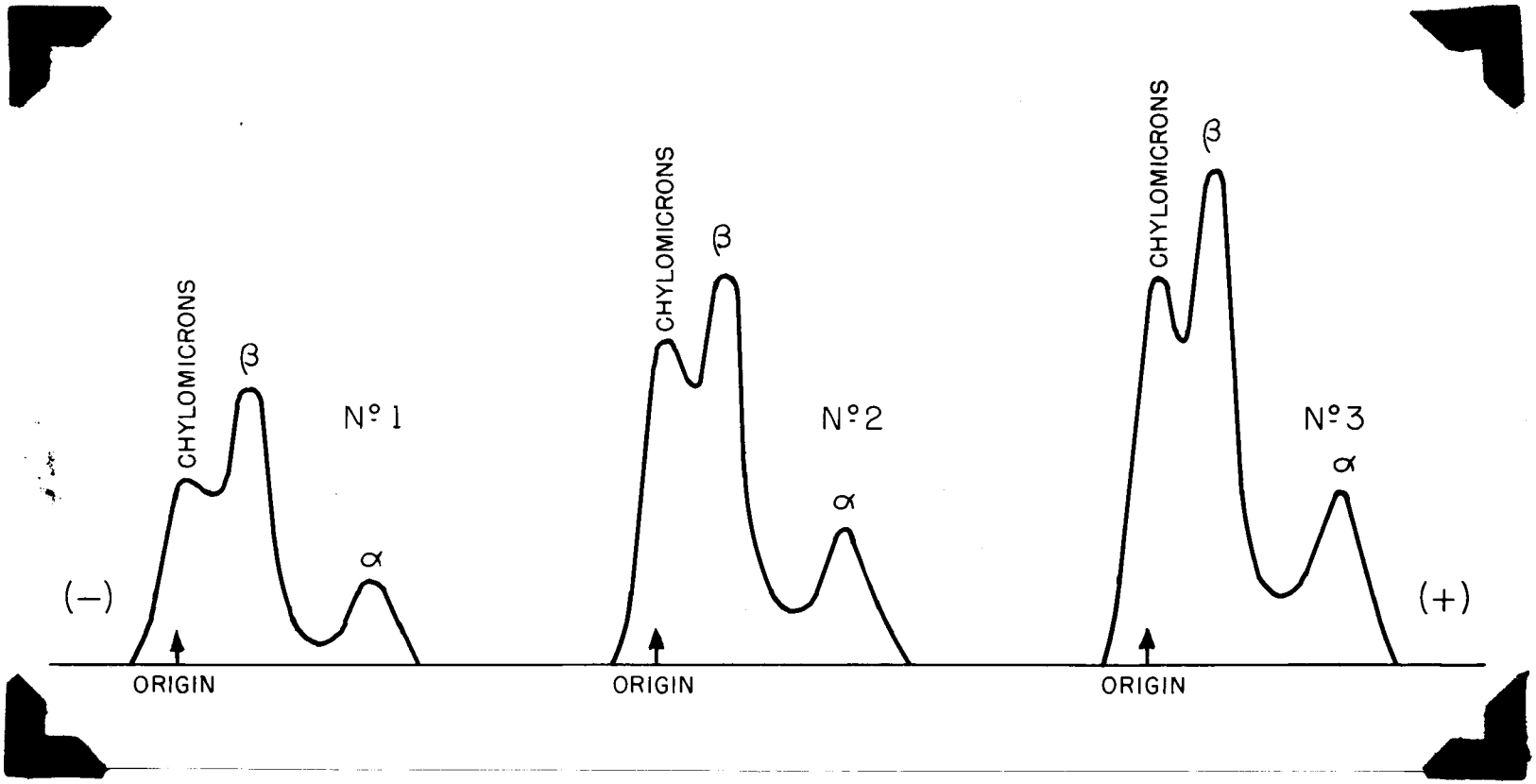

Fig. 4. Scanning of electropherograms of serum lipoproteins. Samples No. 1, No. 2, and No. 3 were pre-stained as indicated in Table I under the same readings.

TABLE II

AREA OF THE DIFFERENT LIPID ZONES IN THE ELECTRO-PHORETIC PATTERNS OF SERUM SAMPLES PRE-STAINED WITH INCREASING AMOUNTS OF DYE SOLUTION

Strip No.	Area (cm^2)			
	Chylomicrons	beta-lipo-proteins	alpha-lipo-proteins	total area
1	1.05	2.50	0.95	4.50
2	2.25	4.50	1.65	8.40
3	3.25	6.85	2.55	12.65
4	5.05	6.25	2.45	13.75

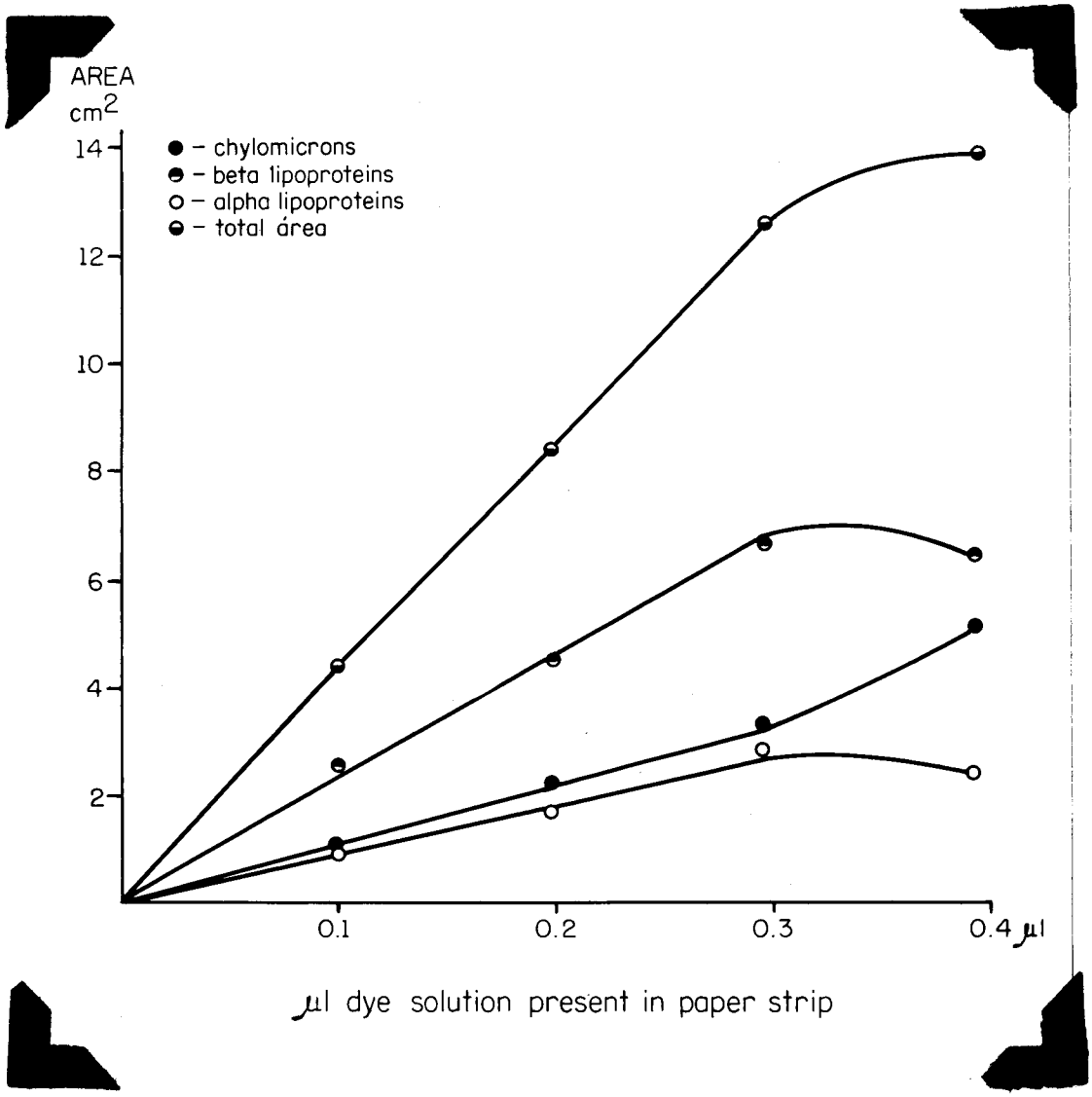

Fig. 5. Effect of the amount of dye solution added to the serum on the color intensity of the lipid zones.

As can be seen from the description of the technique used, increasing amounts of dye solution were added to the same volume of serum. This means that the final volume is not the same in each case and therefore the amount of lipid material present in the aliquot applied to the paper is not the same either. Although the dilution effect is small, we decided to take this fact into consideration in order to have a more accurate and real picture of the effect under investigation. That is the main reason why we have plotted the areas under the peaks against the amount of dye solution present in the paper strip instead of simply plotting it against the amount of dye solution added to the serum.

Interpretation of the curves shown in Figure 4 is reasonably simple. It can be seen that the areas of the zones containing lipids and the total area increase up to the point where 0.296 µl of dye solution are present in the paper strip, that is, when 0.075 ml of the dye solution were added to 0.50 ml of the serum sample. Furthermore, it is also evident that this increase is linear up to this point. Above this concentration, the total area starts to level off and the alpha- and beta-lipoprotein zones actually decrease. Meanwhile, the area corresponding to the chylomicrons starts to rise quite rapidly.

A search for the cause or causes that would explain these facts is not easy to find without leaving a margin of doubt. However, it seems that the reasoning presented below would explain

at least in part, what may be the causes for the behavior illustrated in Figure 5.

It is well known that the presence of organic solvents may upset the equilibrium between the lipid and the protein moiety in the lipid-protein complex. Denaturation of the protein itself may also occur to some extent but the main alteration would be the breakdown of the linkages between the lipid and the protein portion of the complex. This breakdown of bonds would set some of the lipid of the complex free. With less lipid, the alpha- and beta-lipoprotein zones should present smaller areas in the electrophoretic diagrams. On the other hand, the protein-free lipid from the lipoprotein complex would increase the zone of chylomicrons or of neutral fat.

However, it would also seem evident that, in spite of this mechanism, the total area should continue to increase proportionally with the amount of dye solution added to the serum and that the increase in the chylomicrons zone should compensate, somehow, for the decrease in the alpha- and beta-lipoprotein zones.

It is generally accepted that lipid dyes like Sudan Black B color lipids by dissolving in them rather than by a chemical combination. Therefore, the dissolving capacity of the lipids will play an important role in the dyeing process. The partition coefficient of Sudan Black B between propylene glycol and the blood lipids must be much more in favor of the latter and thus Sudan Black B dissolves more readily in the lipids. The level-

ling of the total area would therefore indicate that the equilibrium point was being reached. It is obvious that a linear increase of the total area, or of the lipid fractions, could not hold indefinitely, unless the solubility of Sudan Black B in them was unlimited, which is an unlikely occurrence. Therefore, the levelling of the total area is to be expected.

It also may be of interest to mention that every point in the curve representative of the total area truly corresponds to the sum of the areas for chylomicrons, alpha- and beta-lipoproteins. In view of these facts, the explanation given above seems to be one which fits with the experimental facts, at least for the conditions used in the present investigation.

The remaining question to be analyzed, in order to support our reasoning, was whether the increases in the alpha- and beta-lipoprotein zones were proportional to each other, that is, whether both increase at the same rate.

To verify this relationship, the easiest and quickest way was to determine the beta/alpha ratio. The values obtained for each point in the curve, which have been experimentally determined, are presented in Table III.

The data in Table III show that up to the point where the linear relationship between amount of dye solution present in the paper strip and the area of the lipid zones holds, the beta/alpha ratio is practically the same, within the limits of the experimental error and within the number of significant

TABLE III

BETA/ALPHA RATIO OF THE RUNS ILLUSTRATED IN FIG. 4

Strip No.	Beta/alpha ratio
1	2.7
2	2.7
3	2.7
4	2.5

figures used to calculate the values. However, this value is lower for strip No. 4, where 0.100 ml of dye solution were added to 0.50 ml of serum. This is a clear indication that the increase in alpha- and beta-lipoprotein areas follows the same rate.

In view of the results obtained here, all further experiments were conducted by staining the serum with the same amount of dye solution. To 10 volumes of serum there was added 1 volume of the dye solution, with the precautions mentioned previously.

3. Incubation time of serum with dye solution.

The time of incubation between the serum sample and the dye solution was determined after a series of six experiments. These consisted of incubating aliquots of 0.50 ml of the same serum sample with 0.050 ml of the saturated solution of acetylated Sudan Black B, for different periods of time. Table IV shows how the incubation mixture was prepared.

After the incubation period was over, all tubes were centrifuged simultaneously, as described under general methods. Aliquots of 20 µl. of each preparation were added to the corresponding strip of Macherey and Nagel Nr. 2214ff filter paper. The electrophoretic separations lasted 2 hrs., using a potential gradient of 10 volts per cm., at 25°C.

After the electrophoretic separations, the strips were dried at room temperature, in the dark, and scanned.

TABLE IV

TIME OF INCUBATION BETWEEN SERUM AND DYE SOLUTION.

(ALL TUBES WERE INCUBATED AT 25°C)

Tube No.	ml. of serum	ml. of AcSBB *	Incubation time (minutes)
1	.50	.050	5
2	.50	.050	15
3	.50	.050	25
4	.50	.050	35
5	.50	.050	45
6	.50	.050	60

* Acetylated Sudan Black B.

The areas obtained by planimetry of the scanning curves for alpha- and beta-lipoproteins are shown in Table V, where the strip numbers correspond to tube numbers presented in Table IV.

It is evident from the data in Table V, that the incubation for 35, 45 and 60 minutes gives practically the same results.

For shorter periods of incubation, the results are quite different. The beta/alpha ratio was also calculated for each case and the results obtained are presented in Table VI.

The values were calculated since it was possible that the beta/alpha ratio for the first three strips was the same as for those in which longer periods of incubation were used, although the individual values were quite different. The data in Table VI shows that even the beta/alpha ratio differs markedly from those obtained for the three last strips.

In Fig. 6 the results of these experiments are shown in order to present more clearly the region where constant values are obtainable. The smaller inner graph represents the beta/alpha ratio variations.

One may conclude that the minimum time of incubation between the serum sample and the dye solution should be about 35 minutes. To make sure that a perfect equilibrium between the serum and the dye solution has been attained, we have selected an incubation period of 45 minutes for all subsequent experiments.

In a few instances, the serum and the dye solution were

TABLE V

AREA OF THE ALPHA- AND BETA-LIPOPROTEIN FRACTIONS AFTER INCUBATING THE SERUM WITH THE DYE SOLUTION FOR DIFFERENT PERIODS OF TIME

Strip No.	Area (cm^2)		Incubation time (min.)
	alpha-lipoproteins	beta-lipoproteins	
1	0.50	2.61	5
2	0.55	2.60	15
3	0.50	2.70	25
4	0.84	3.30	35
5	0.83	3.28	45
6	0.85	3.31	60

TABLE VI

BETA/ALPHA RATIO OBTAINED FOR THE RUNS ILLUSTRATED IN TABLE V

Strip No.	Beta/alpha ratio
1	5.22
2	5.00
3	5.40
4	3.93
5	3.95
6	3.89

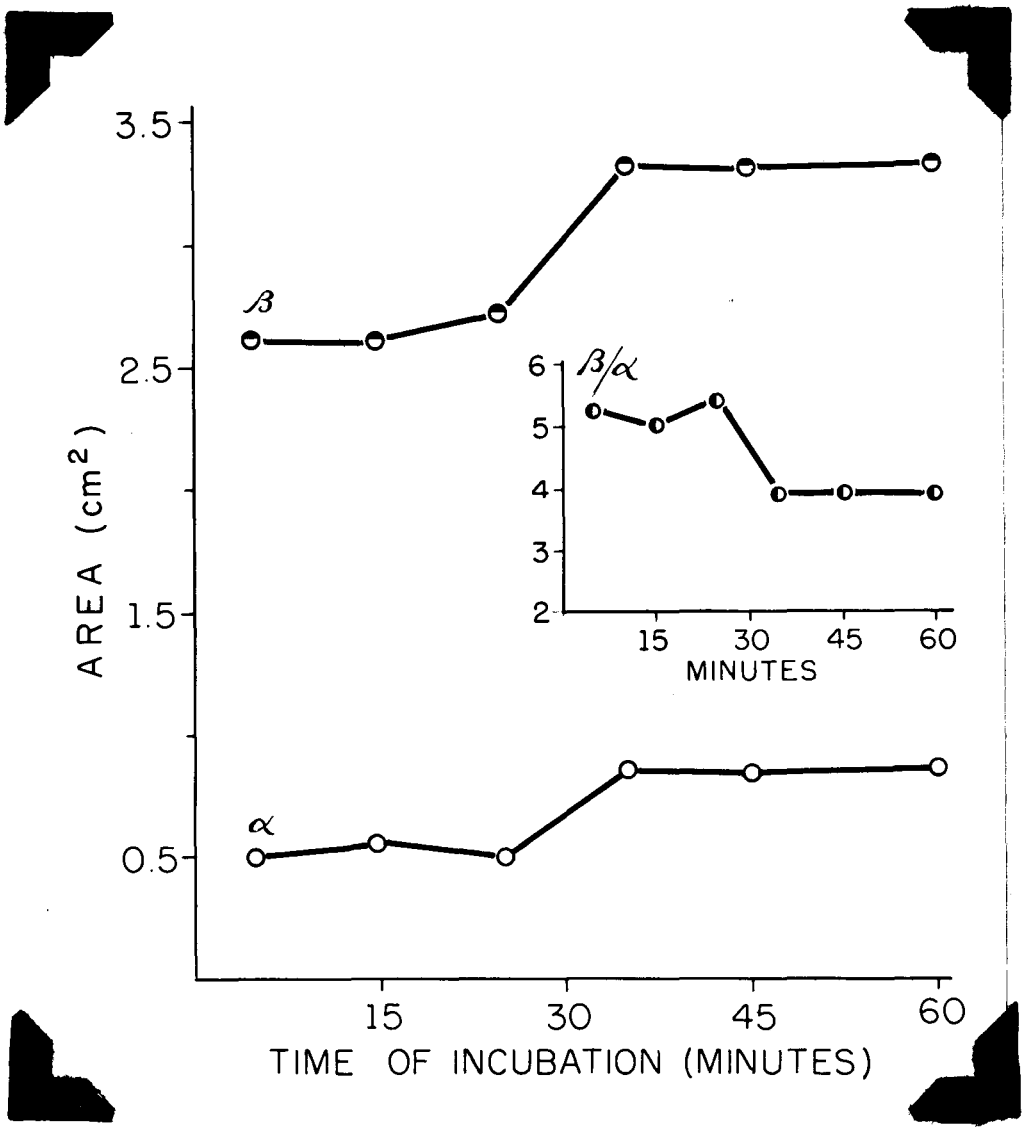

Fig. 6. Variation of lipoproteins and of the beta/alpha ratio with the time of incubation.

incubated at 37°C for 30 minutes and 45 minutes, respectively. The results obtained clearly showed that there is no improvement when the incubation mixture is kept at 37°C. This temperature has been proposed by Larkey and Belko(41) but since no improvement in the results were noted, we decided to avoid it in our experiments. Also, increasing the temperature may alter the protein-lipid complex which is sensitive to heat. At 25°C no visible alterations could be detected by paper electrophoresis.

4. Amount of stained serum to be added to the paper.

After establishing the conditions already discussed, we decided to check the dependence of the amount of pre-stained serum in the strip with Beer's law. This could give us an idea of the proper amounts to be used in routine determinations of the alpha- and beta-lipoprotein fractions and also permit us to determine whether the method could be used on a semi-quantitative basis.

This dependence was studied by a series of six experiments in which different aliquots of the same pre-stained serum were applied to the paper strips. To 0.50 ml of each of the six serum samples used, 0.050 ml of the acetylated Sudan Black B solution were added and the mixture incubated at 25°C for 45 minutes. After centrifuging, aliquots of 5, 10, 20, 30, 40 and 50 µl were used for the electrophoretic fractionation. In this particular type of experiment, we have used preferably Whatman 3MM filter paper strips since they are thicker and can, therefore, hold

more solution. The electrophoretic separations lasted 2 hours and 30 minutes at a potential gradient of 7,8 volts/cm.

After the electrophoretic runs, the strips were air-dried in the dark as usual, and scanned. The values obtained by planimetry of the areas for the alpha- and beta-lipoproteins are presented in Table VII, together with the beta-alpha ratio for each case.

The values thus obtained were then plotted on a graph against the volume of pre-stained serum applied to the paper strips as shown in Fig. 7.

This graph clearly shows that there is a linear increase of the area between the points where 10 µl and 40 µl of the pre-stained serum were applied to the paper strips. Increasing the volume of serum above 40 µl caused a variation from linearity, under the experimental conditions described. The beta/alpha ratio was practically constant throughout the range where linearity holds. Furthermore, it should be noted that the linear increase observed follows a straight line passing through the origin of coordinates.

Kanabrocki et al.(34), in similar experiments also found a linear increase of integration units of their scanning instrument with increasing amounts of dyed serum applied to the paper strips, between 10 µl and 60 µl. However, the lines obtained, both for alpha- and beta-lipoproteins do not pass through zero. Also, these authors stated that the beta/alpha lipoprotein

TABLE VII

VARIATION OF THE AREA FOR THE ALPHA- AND BETA-LIPOPROTEINS WITH INCREASING VOLUMES OF PRE-STAINED SERUM

Volume of serum: µl	Area (cm^2)		Beta/alpha ratio
	Alpha-lipoproteins	Beta-lipoproteins	
5	0.50	0.75	1.50
10	0.75	1.95	2.60
20	1.45	3.75	2.59
30	2.50	5.80	2.58
40	3.10	8.15	2.63
50	3.60	9.00	2.50

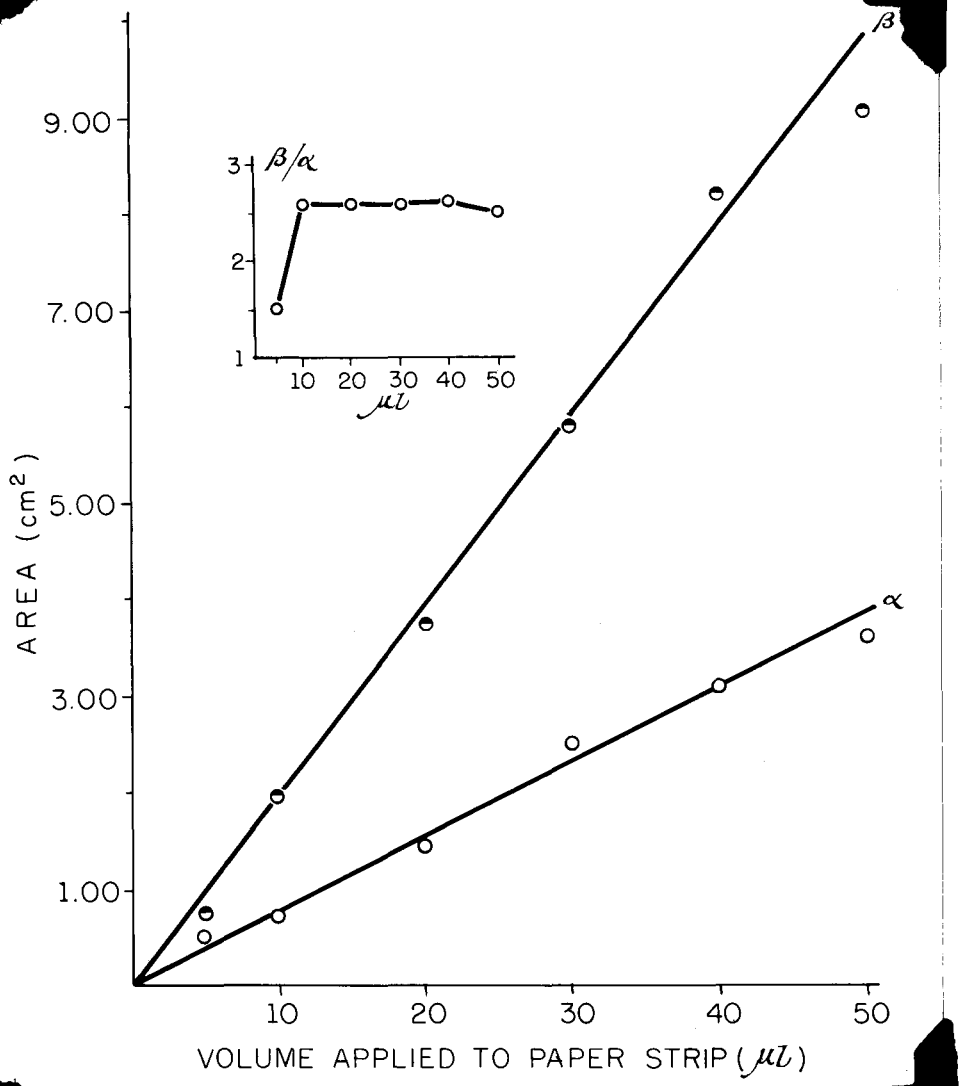

Fig. 7. Variation of the Area of lipoprotein fractions with the volume of pre-stained serum applied to the paper strip.

ratio was relatively constant in this range. Rough calculations from their graph showed that the ratio varies somewhere from 1.1 to 2.4, which is a quite high variation for the same serum sample.

The results we have obtained here clearly indicate that the method as described is suitable for semi-quantitative determinations, since the color of the lipoprotein zones follows Beer's law when volumes varying from 10 µl to 40 µl are used. We have selected 20 µl as the best volume to be used in further experimental determinations.

5. Stability of the color of the stained lipoproteins.

It is known that the stained lipoproteins are extremely sensitive to light(34). In order to evaluate the effect of light on the stained lipoproteins, six serum samples were run simultaneously. These samples were pre-stained using the conditions selected in the previous experiments.

For the electrophoretic separations, 20 µl of each pre-stained serum were applied to Mecherey & Nagel No. 2214ff filter paper strips. A potential gradient of 8 volts/cm was used and the run lasted for two hours.

After the electrophoretic separations, the strips were immediately removed and dried at 50°C, in the dark. As soon as they were dried they were scanned and separated into two groups of three strips each. One group was kept in the dark all the time and the other group was left exposed to the ordinary light

of the laboratory (daylight and artificial light), except nights. The strips of both groups were scanned periodically.

The results of these experiments are shown in Tables VIII and IX, in which the values reported represent the mean values obtained for the 3 strips of each group after duplicate individual determinations. Since we were only concerned with the effect of light, no other possible causes for variations were tested.

As can be seen from the data presented in Tables VIII and IX there is a marked fall in the intensity of the color of the stained lipoproteins when the strips are left exposed to light. On the other hand, the values are practically constant for the strips which have been kept in the dark. Since the observed fall in color in those strips which have been left exposed to light could be somewhat proportional for each individual fraction, the beta/alpha ratio was calculated and included in the tables.

The effect of light on the stained lipoproteins can be more clearly seen when the results are expressed as per cent of the original readings. This data is presented in Tables X and XI.

The values for the total area were plotted on a graph as shown in Fig. 8. Since the total area represents the total color in the strips, only this value was plotted to illustrate the effect of light on the stained lipoproteins. The color change in the strips exposed to light can readily be appreciated

TABLE VIII

AREA (cm^2) OF THE LIPOPROTEIN FRACTIONS, TOTAL AREA, AND THE BETA/ALPHA RATIO FOR THE STRIPS KEPT IN THE DARK

Fraction	Hours kept in the dark					
	0	1,5	3	17,5	25,5	50
alpha	2.47	2.53	2.53	2.43	2.50	2.43
beta	3.83	3.87	3.90	3.70	3.80	3.77
chylomicrons	2.87	2.83	2.97	2.93	2.77	2.77
total area	9.17	9.23	9.40	9.06	9.07	8.97
beta/alpha	1.55	1.53	1.54	1.52	1.52	1.55

TABLE IX

AREA (cm^2) OF THE LIPOPROTEIN FRACTIONS, TOTAL AREA, AND THE BETA/ALPHA RATIO FOR THE STRIPS EXPOSED TO LIGHT

Fraction	Hours exposed to light				
	0	1	2	17	25
alpha	2.40	2.27	1.90	1.73	1.67
beta	3.83	3.80	3.43	3.07	2.47
chylomicrons	2.43	2.13	1.93	1.97	1.80
total area	8.66	8.20	7.26	6.77	5.94
beta/alpha	1.60	1.67	1.81	1.77	1.48

TABLE X

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS KEPT IN THE DARK

Fraction	Hours kept in the dark					
	0	1.5	3	17.5	25.5	50
alpha	100	102.4	102.4	98.4	101.2	98.4
beta	100	101.0	101.8	96.6	99.2	98.4
chylomicrons	100	98.6	103.5	102.1	96.5	96.5
total area	100	100.7	102.5	98.8	98.9	97.8

TABLE XI

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS EXPOSED TO LIGHT

Fraction	Hours exposed to light				
	0	1	2	17	25
alpha	100	94.6	79.2	72.1	69.5
beta	100	99.2	89.6	80.2	64.5
chylomicrons	100	87.7	79.4	81.1	74.1
total area	100	94.7	83.8	78.2	68.5

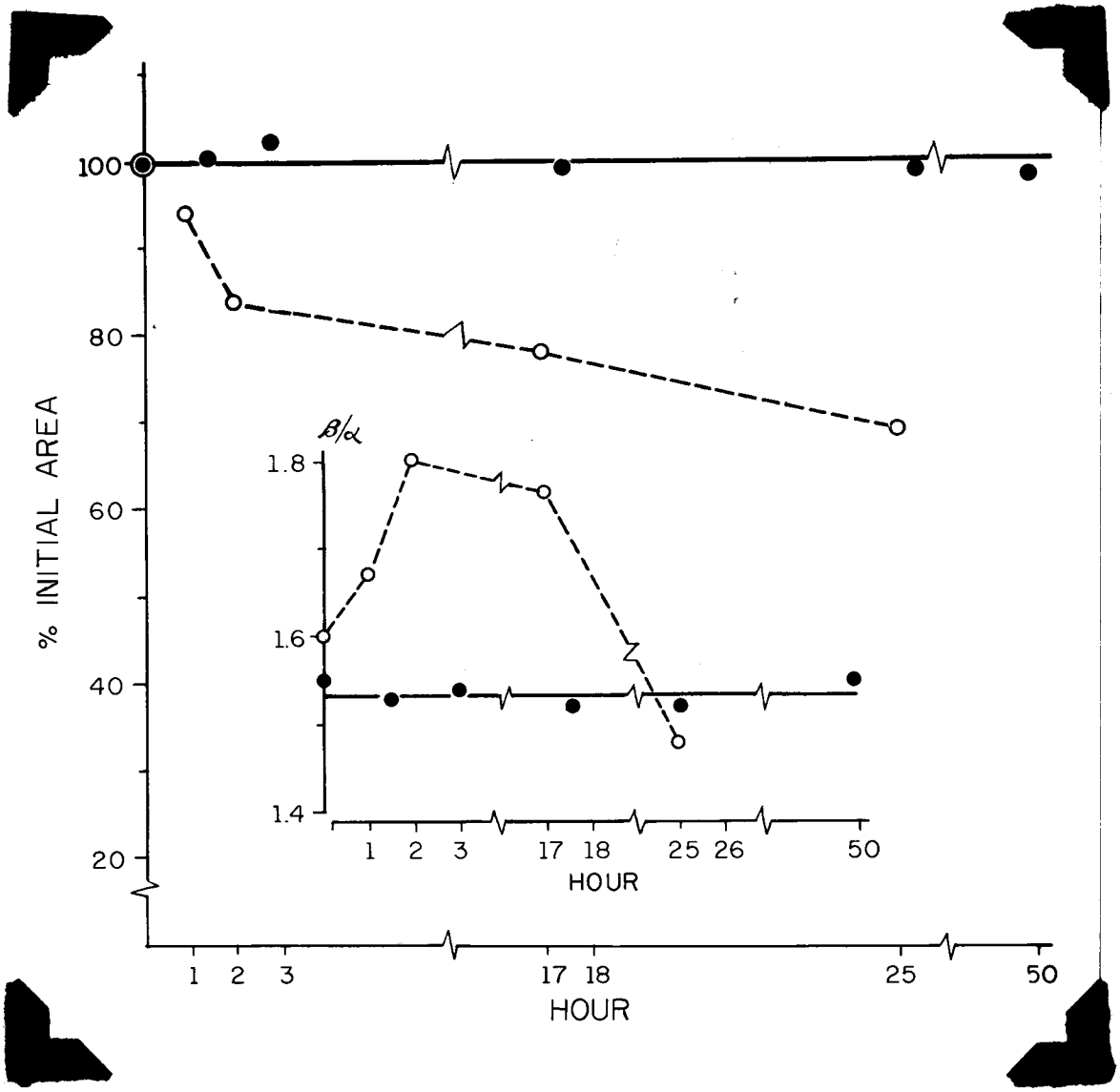

Fig. 8. Total area for lipoproteins in strips exposed to light (-----) and in strips kept in the dark (———).

in Fig. 8. The small inner graph represents the variation in the beta/alpha ratio in each case, in order to show that the change in color is not proportional to each individual fraction.

From these experiments it is concluded that the stained lipoproteins are very sensitive to light. However, the color in the lipidograms is perfectly stable, at least for 50 hours, when the strips are kept in the dark. It is therefore proposed that strips should be kept in the dark and that readings should be made within 48 hours after the completion of the run.

6. Proposed technique.

In view of the results so far discussed, the following technique is proposed for the determination of serum lipoproteins by paper electrophoresis, using pre-stained serum.

a. Staining solution:

10 ml propylene glycol (analytical grade) are heated to 100°C and 0.1 g of acetylated Sudan Black B (Hartman-Leddon Co., Philadelphia, Pa.) is added with through stirring for 5 minutes. The solution is filtered hot on Whatman No. 2 filter paper, cooled and re-filtered on the same kind of paper.

b. Pre-staining:

The staining solution is added to fresh serum, at room temperature, with constant gentle stirring, in the ratio of 1:10 v/v, respectively. The serum-dye mixture is left at room temper-

ature (25°C) for 45 minutes and then centrifuged to remove the excess dye, if any (2.500 r.p.m. for 15 minutes in an international Clinical Centrifuge Model CL is most convenient). The supernatant is used for the electrophoretic separation.

c. Paper electrophoresis:

The technique and instrument for the electrophoretic separations have been described under General Methods. The recommended procedure is as follows; the pre-stained serum (20 ul) is added as a thin streak across the width of Macherey & Nagel No. 2214 ff paper strips (2.5 cm x 33 cm in our apparatus) midway between the ends. A potential gradient of 8 volts/cm is applied for two hours, at 25°C, which gave a current of 1.5 mA/strip at the end of the experiment, under our experimental conditions. Separations are carried out using veronal buffer, pH 8.6, ionic strength of 0.05. After completion of the run the strips are removed and dried immediately in a horizontal position, at 50°C, in the dark. Duplicate runs of individual samples are always made.

d. Scanning:

The dried strips are kept in the dark and scanned at 595 mμ within 48 hours. Quantitation of the diagrams can be made by planimetry, self-integrating scanners or by elution. Since in routine determinations one is concerned only with alpha- and beta-lipoproteins, only these fractions are determined and the results are expressed as percentages of the sum of these two fractions.

The beta/alpha ratio is calculated from these values.

The method described here can be adapted to any particular set of conditions. McDonald and Kissane(50), for instance, have proposed a solubilizing procedure for the preparation of Sudan Black B solutions which has been used successfully(47). Also, they recommend that pre-staining should be done using a 1:5 v/v ratio between dye solution and serum, respectively. This procedure should be most useful especially for serum samples with a low lipoprotein content.

Naturally, the electrophoretic technique can be varied in many ways, according to the individual conditions and laboratory facilities. Larger strips of filter paper were tried and gave very good results. In this last case, volumes greater than 20 µl may be applied to the paper. Although we have used Whatman 3MM filter paper with success, improved separations have always been obtained by the use of Macherey & Nagel No. 2214 ff paper strips.

The color stability is very good when the strips are kept in the dark as mentioned previously. However, it should be mentioned that light alone is not the sole cause for color changes of the stained lipoproteins. We have also observed that when the strips are covered with transparent cellophane tape the intensity of staining remains for longer periods of time even when the strips are exposed to light. This observation agrees with the recommendations of Kanabrocki *et al.*(34) who have dried the strips

in the dark and then sprayed them with a coat of acrylate, and finally covered them with tape. According to them, the acrylic coat alone is sufficient for maintaining the color stable for many months.

With any kind of modification in instrument and conditions the method can be used satisfactorily, as long as the necessary precautions are taken. As described here, the method is shown to be reproducible and to have a good precision, as we shall see next.

7. Reproducibility and error of the method.

After describing the proposed technique it became necessary to check the reproducibility and to determine the error of the method. For this purpose, seven samples of the same serum were individually pre-stained and run in two separate sets, on two consecutive days. The electrophoresis was performed with the recommendations described in the preceeding section, using Macherey & Nagel No. 2214 ff filter paper strips. The usual 8 volts/cm potential gradient was used for a period of two hours.

After electrophoresis, the strips were immediately dried in the dark, and scanned. Table XII shows the results obtained after planimetry of the areas. The results are expressed as percentages of the total area, which includes the chylomicrons, in this particular case. The beta/alpha ratio for each determination is also shown.

TABLE XII

PER CENT COMPOSITION OF ALPHA-, BETA-LIPOPROTEINS, CHYLO-MICRONS AND THE BETA/ALPHA RATIO FOR 7 RUNS OF THE SAME SERUM

Run	Per cent composition			beta/alpha
	alpha	beta	chylomicrons	
1	28.71	40.60	30.69	1.41
2	28.42	41.05	30.53	1.44
3	27.84	41.24	30.92	1.48
4	27.06	43.71	29.23	1.62
5	26.88	40.86	32.26	1.52
6	29.07	40.69	30.24	1.40
7	28.67	41.11	30.22	1.43
Mean±S.D.	28.09±0.85	41.32±1.08	30.58±0.91	1.47±0.08

$S.D. = \pm \left(\sum (x-\bar{x})^2/(n-1) \right)^{1/2}$

As can be noticed, the reproducibility of the method is quite satisfactory and the standard deviation (S.D.) for each fraction and for the beta/alpha ratio is very small. From this data presented in Table XII we were able to calculate the coefficient of variation for each fraction, which permitted us to have an idea of the error of the method. In Table XIII, these variations are expressed as percentages, which represent the error of the method.

The data in Table XIII shows that the results obtained by the method described are quite reproducible and that they are within ± 3.0 per cent for each individual fraction. The beta/alpha ratio showed a reproducibility of results which is within ± 5.4 per cent. The method can therefore be considered to have a good precision and reproducibility.

TABLE XIII

COEFFICIENT OF VARIATION FOR THE VALUES OF THE DIFFERENT LIPOPROTEIN FRACTIONS, EXPRESSED AS PERCENTAGES

Fraction	Variation
alpha	± 3.0 %
beta	± 2.6 %
chylomicrons	± 3.0 %
beta/alpha	± 5.4 %

CHAPTER IV

LIPOPROTEIN LEVELS IN EXPERIMENTAL LIVER DAMAGE

It is known that carbon tetrachloride causes liver damage. This impairement of the liver is followed by an accumulation of fat in the liver, probably because CCl_4 poisons the secretory mechanism by which the liver is constantly secreting large amounts of triglycerides into the plasma(65).

The liver has been shown to be the site of plasma cholesterol and phospholipid synthesis(29)(40). Later evidence that the liver is capable of synthesizing plasma lipoproteins was furnished by Miller *et al.*(52) and more recently, by Marsh and Whereat(44).

These findings, and those mentioned previously in earlier sections of this thesis, suggested the present study of the serum lipoprotein levels of rats poisoned with carbon tetrachloride.

1. Control Experiments.

A group of 12 male Wistar white rats, weighing between 100 and 150g was selected for these experiments. Since dietary protein quality may alter the serum lipoprotein patterns(25), the animals were kept in the laboratory on the same well balanced standard diet for at least two weeks prior to use. The animals were fed and watered *ad libitum* during this period of time.

After this adaptation period, the control animals were injected subcutaneously with 0.1 ml isotonic saline/100 g body weight. They were than starved for 12-24 hours in order to achieve a metabolic equilibrium in all animals.

Blood samples were withdrawn by heart puncture under light ether anesthesia and the serum separated as described under general methods.

Lipoprotein patterns were obtained using the method described in Chapter III. The mean values obtained after duplicate determinations of each individual sample for the alpha- and beta-lipoproteins are shown in Table XIV.

Table XIV also shows the values for beta/alpha ratio, calculated for each case. The lower figures represent the mean plus or minus standard deviation obtained for each lipoprotein fraction and for the beta/alpha ratio in this group of animals.

2. Effect of CCl_4 on the lipoproteins of rats.

For these experiments, a group of 8 rats was used. After keeping the animals on the standard diet for 3 weeks, all 8 animals were injected subcutaneously with 0.1 ml of CCl_4 per 100 g body weight. The animals were starved immediately after the injection and blood samples collected 24 hours later. The results obtained for the serum lipoproteins and for the beta/alpha ratio in these cases are shown in Table XV.

TABLE XIV

PER CENT COMPOSITION OF ALPHA- AND BETA-LIPOPROTEINS, AND THE BETA/ALPHA RATIO IN SERUM OF CONTROL RATS

Animal Nº	Alpha-lipoproteins	Beta-lipoproteins	Beta/alpha ratio
1	60.6	39.4	0.65
2	57.0	43.0	0.75
3	54.8	45.2	0.82
4	56.6	43.4	0.77
5	53.9	46.1	0.86
6	53.1	46.9	0.88
7	50.6	49.4	0.98
8	54.9	45.1	0.82
9	51.1	48.9	0.96
10	54.3	45.7	0.84
11	54.8	45.2	0.82
12	53.5	46.5	0.87
Mean ± s.d.	54.6 ± 2.7	45.4 ± 2.7	0.84 ± 0.09

$$\text{s.d.} = \pm \left[\sum (x - \bar{x})^2 / (n - 1) \right]^{1/2}$$

TABLE XV

LIPOPROTEIN LEVELS OF RATS INJECTED WITH CCl_4, 24 HOURS AFTER THE ADMINISTRATION OF THE DRUG

Animal No.	Alpha (%)	Beta (%)	Beta/ Alpha
13	20.8	79.2	3.82
14	30.6	69.4	2.27
15	25.6	74.4	2.91
16	33.2	66.8	2.01
17	24.3	75.7	3.12
18	28.4	71.6	2.52
19	31.2	68.8	2.21
20	30.0	70.0	2.23
Mean ± s.d.	28.0 ± 4.1	72.0 ± 4.1	2.64 ± 0.60

As can be seen in Table XV, there is a great increase in the percentage values for the beta-lipoproteins 24 hours after the injection of CCl_4. Because of this fact, we became interested in knowing whether the change was permanent or transitory.

For this study, a group of 30 animals was used. All the animals were kept on the standard diet, as before and injected with the same dosage of CCl_4 i.e., 0.1 ml per 100 g body weight. Blood samples were collected from groups of animals successively at 48, 72, 96 and 120 hours after the injection.

Each group was starved for 24 hours before bleeding and fed the standard diet the rest of the time. Two samples, one belonging to the 48 hours group and other from the 72 hours group were discarded, since they exhibited slight signs of hemolysis.

The results obtained for the lipoprotein levels in these groups are presented in Tables XVI, XVII, XVIII and XIX.

The results obtained were analyzed statistically in order to determine whether the increase was significant or not. Since only relative percentages are involved, analysis of individual fractions are not necessary. The changes are reflected in the beta/alpha ratio. An increase in the value of this ratio would have to indicate an increase in the relative amount of the beta component and vice-versa. Only the beta/alpha variations were, therefore, taken into consideration.

It may be of interest to state that the significance of each individual mean for the alpha- and beta-lipoproteins and

TABLE XVI

LIPOPROTEIN LEVELS OF RATS INJECTED WITH CCl_4, 48 HOURS AFTER THE ADMINISTRATION OF THE DRUG

Rat No.	Alpha (%)	Beta (%)	Beta/ Alpha
21	24.8	75.2	3.03
22	31.6	68.4	2.16
23	24.0	76.0	3.17
24	34.2	65.8	1.92
25	27.1	72.9	2.69
26	30.0	70.0	2.33
27	-	-	-
Mean ± s.d.	28.6 ± 4.0	71.4 ± 4.0	2.55 ± 0.50

TABLE XVII

LIPOPROTEIN LEVELS OF RATS INJECTED WITH CCl_4, 72 HOURS AFTER THE ADMINISTRATION OF THE DRUG

Rat No.	Alpha (%)	Beta (%)	Beta/ Alpha
28	32.0	68.0	2.13
29	29.1	70.9	2.44
30	-	-	-
31	36.2	63.8	1.76
32	26.2	73.8	2.82
33	33.4	66.6	1.99
34	26.8	73.2	2.73
Mean ± s.d.	30.6 ± 3.9	69.4 ± 3.9	2.31 ± 0.42

TABLE XVIII

LIPOPROTEIN LEVELS OF RATS INJECTED WITH CCl_4, 96 HOURS AFTER THE ADMINISTRATION OF THE DRUG

Rat No.	Alpha (%)	Beta (%)	Beta/ Alpha
35	28.8	71.2	2.47
36	33.6	66.4	1.98
37	27.6	72.4	2.62
38	33.0	67.0	2.03
39	29.8	70.2	2.36
40	31.4	68.6	2.18
41	38.0	62.0	1.63
Mean ± s.d.	31.7 ± 3.5	68.3 ± 3.5	2.18 ± 0.34

TABLE XIX

LIPOPROTEIN LEVELS OF RATS INJECTED WITH CCl_4, 120 HOURS AFTER THE ADMINISTRATION OF THE DRUG

Rat No.	Alpha (%)	Beta (%)	Beta/ Alpha
42	43.1	56.9	1.32
43	42.3	57.7	1.36
44	39.5	60.5	1.53
45	40.1	59.9	1.49
46	41.5	58.5	1.41
47	36.0	64.0	1.78
48	37.2	62.8	1.69
49	49.9	50.1	1.00
50	38.8	61.2	1.58
Mean ± s.d.	40.9 ± 4.0	59.1 ± 4.0	1.46 ± 0.23

for the beta/alpha ratio was tested. This was accomplished in a simple way by dividing the mean by its standard deviation and looking up the corresponding probability in the proper tables. We have used Student's "t" test in all the cases. In testing to see whether the individual means differed significantly from zero or not, we have always found values lying between $P < 0.001$ and $P < 0.01$. This was a clear indication that the means were highly significant.

Testing the significance of the difference of two means we have used the following formulas:

$$t = \frac{\bar{x} - \bar{x}'}{S_D} \qquad \text{and} \qquad S_D = \left[\frac{S_{dx}^2}{N_1} + \frac{S_{dx'}^2}{N_2} \right]^{1/2}$$

where: S_D = the standard deviation of the difference of the means $\bar{x}$ and $\bar{x}'$,

S_{dx} and $S_{dx'}$ = the standard deviation of the first and second samples, respectively,

N_1 and N_2 = the number of items in the first and second samples, respectively.

(See: Arkin, H. and Colton, R. R., "Tables for Statisticians", p. 14, Barnes and Noble, Inc., New York, 1953 and "Outline of Statistical Methods", 4th. Edition, p. 121, Barnes & Noble, Inc., N. Y., 1942).

Table XX shows the results obtained in the analysis of

TABLE XX

ANALYSIS OF THE VARIATION OF THE BETA/ALPHA RATIO OF RATS INJECTED WITH CCl_4

Time	No. of Animals	Alpha Mean ± s.d.	Beta Mean ± s.d.	Beta/Alpha Mean ± s.d.	P to normal (β/α)	P to 24 hrs (β/α)	P to 96 hrs (β/α)
0	12	54.6 ± 2.7	45.4 ± 2.7	0.84 ± 0.09	-	-	-
24	8	28.0 ± 4.1	72.0 ± 4.1	2.64 ± 0.60	< .001	-	-
48	6	28.6 ± 4.0	71.4 ± 4.0	2.55 ± 0.50	< .001	> .80	-
72	6	30.6 ± 3.9	69.4 ± 3.9	2.31 ± 0.42	< .001	> .30	-
96	7	31.7 ± 3.5	68.3 ± 3.5	2.18 ± 0.34	< .001	> .10	-
120	9	40.9 ± 4.0	59.1 ± 4.0	1.46 ± 0.23	< .001	> .001	< .001

the results for the beta/alpha ratio. As one can see, the ratio is increased in all groups of rats injected with CCl_4. Furthermore, it is evident that the maximum increase was found 24 hours after the injection.

The value starts falling slightly but the statistical analysis showed that the fall was only significant, 120 hours after the injection. In fact, the fall of the value for 120 hours is even statistically different from the value for 96 hours.

The behavior analyzed above can be seen in Fig. 9 where the range of variations are pictured. There is a visible fall in the value for the beta/alpha ratio 120 hours after the injection as compared to the value obtained 24 hours after the administration of CCl_4. Nevertheless, this value at 120 hours is still increased as compared with the value obtained for control animals.

Fig. 9. Range variations of the beta/alpha ratio in control rats and in animals injected with a single dose of CCl_4.

CHAPTER V

DISCUSSION AND CONCLUSIONS

To begin with, some final comments on the proposed method for the determination of serum lipoproteins are in order.

First, it would be advisable to comment on what is being determined by this method. Based on ultracentrifugal analysis and density determinations, the human serum lipoproteins are generally classified into 4 groups or classes of lipoproteins and the chylomicrons which have a very low protein content(42) (58).

By paper electrophoresis, using veronal buffer of pH 8.6, it is not possible to separate the 4 groups from the chylomicrons. Usually, only two fractions are obtained; i.e. the alpha- and the beta-lipoproteins. The relationship between electrophoretical findings and the results obtained by ultracentrifugation is shown in Table XXI.

As shown in Table XXI, what is called beta-lipoproteins in the present investigation correspond to the lipoproteins having a Sf value of 0-400. The alpha-lipoproteins correspond to the high density lipoproteins. The chylomicrons have the same designation as in ultracentrifugal analysis and correspond to lipoproteins having a Sf value greater than 400. This correspondence between paper electrophoretic and ultracentrifugal analysis has

TABLE XXI

RELATIONSHIP BETWEEN ULTRACENTRIFUGAL AND ELECTROPHORETIC ANALYSIS OF HUMAN SERUM LIPOPROTEINS

Density	S_f d = 1,063	Electrophoretic mobility (free solution)	Designation in present investigation (paper electrophoresis)
0.94	> 400	variable	chylomicrons
0.98	400-20	beta$_1$-or alpha$_2$-globulin	beta-lipoproteins
1.03	20-0	beta$_1$-globulin	
1.09	< 0	alpha$_1$-globulin	alpha-lipoproteins
1.14	< 0	alpha$_1$-globulin	

been confirmed recently by Pezold, DeLalla and Gofman(61).

The proposed method seems to be very useful in the determination of these lipoproteins. The error of the method is low and the reproducibility is very good as shown in the preceding chapter. Some authors prefer to determine the lipoproteins based on their cholesterol content(23). This technique may lead to false conclusions in some instances. Blohm, Kariya and Laughlin (10) showed that 1-[(4-diethylaminoethoxy)phenyl]-1-(p-Tolyl)-2-(p-Chlorophenyl)ethanol (MER-29) is able to inhibit cholesterol biosynthesis. Further, they showed that this compound reduces the cholesterol/phospholipid ratio and also reduces the cholesterol content of lipoproteins. However, it does not change the total amount of lipoproteins as determined by fat soluble dyes. In view of these facts the determination of cholesterol alone may lead to false results in the estimation of total lipoproteins.

The use of dyes still seems to be the more adequate method in paper electrophoresis of serum lipoproteins. Pre-staining offered real advantages over other methods, as has already been shown. The pre-staining technique has been accepted rather generally and has even been proposed in the ultracentrifugal analysis of lipoproteins(21).

The proposed method can therefore be applied successfully to the determination of lipoprotein levels as we have shown in the case of liver poisoning by carbon tetrachloride.

The most important finding in this case was the increase

in the beta-lipoprotein levels in rats poisoned with 0.1 ml of CCl_4 per 100 g body weight. This increase is observed 24 hours after the administration of the drug and remains at higher levels for approximately 96 hours. It starts falling 120 hours after the injection but still is above control levels at this time.

This increase can be visualized through the variations in the analysis of the beta/alpha ratio. There is a marked increase in the values of this ratio which persists steadily for about 96 hours. After that period of time the values start returning to those observed in the normal controls.

This tendency of the beta/alpha ratio to return to normal control levels probably reflects a regeneration of the damaged liver. It is known that carbon tetrachloride, at the dose indicated, produces a lesion that is perfectly reversible. Also, if one considers the fact that the diet supplied contains small amounts of lipotropic agents, especially choline, this regeneration of the liver is to be expected.

It is known that in some forms of hepatic disease (as in biliary cirrhosis) the distribution of lipids and the lipid composition of the lipoprotein fractions may vary form the normal patterns. Usually, there is an increased content of cholesterol (71). Since cholesterol is chiefly transported by beta-lipoproteins the increase of this fraction found in our investigation may reflect an increase in cholesterol esters in rats poisoned by carbon tetrachloride.

These results confirm previous findings that there is an elevation of serum lipids in the initial stage of carbon tetrachloride poisoning. This initial elevation of serum lipids is somewhat parallel to the increased content of fat in the depots as demonstrated by histopathological techniques by several authors (35) (76).

The increase in serum lipids (in terms of the lipoproteins involved) is probably due to impairment of the degradation mechanisms, for fatty acids but certainly not of the mechanism responsible for the mobilization of fat from the liver to the depots.

It is interesting to mention that Pierce and Gofman(62) showed by ultracentrifugation analysis that CCl_4 is able to produce a marked increase in all classes of lipoproteins (S_f 3-12, 12-20 and 20-40) in rabbits injected with the drug, and that these classes correspond to the beta-lipoproteins of the present investigation.

Summarizing the observations of several authors(15)(64) it seems rather convincing that uncoupling of oxidative phosphorylation or failure of fat oxidation could not alone account for the early rise in liver triglyceride content during CCl_4 poisoning. Further, the work of Byers and Friedman(14) clearly demonstrates that the liver is capable of secreting large amounts of triglycerides into the plasma. Therefore, it seems that CCl_4 acts by poisoning this secretory mechanism.

It also seems that the high levels of serum beta-lipoproteins could be a consequence of this fat and cholesterol accumulation in the liver. The inability of the damaged liver to handle endogenous or exogenous fats and cholesterol would increase the synthesis of protein-lipid complexes and also their degradation by an obscure mechanism. However, one still may not exclude the possibility of other organs or systems being involved in the lipoprotein metabolism in this abnormal state, although it has been demonstrated that the "normal" liver is able to synthesize serum lipoproteins.

As found by many investigators(76)(77), CCl_4 produces a marked increase in the concentrations of serum beta-globulins. Our findings strongly suggest that this rise in the beta-globulins is probably due to increased lipoprotein concentrations.

Finally, one may conclude that CCl_4 causes an elevation of serum beta-lipoproteins probably as a result of fat accumulation in the liver in the early stages of this intoxication. The well known fact that the failure of the hepatic secretory mechanism causes a fall in plasma triglycerides and a corresponding elevation of liver triglycerides suggests that CCl_4 poisons this secretory mechanism. The elevated fat and cholesterol content of the liver would increase the rate of synthesis of beta-lipoprotein as an alternative to eliminating the excess fat and cholesterol from the liver.

CHAPTER VI

SUMMARY

1. In the present paper, there is described a simple technique for the determination of serum lipoproteins by paper electrophoresis. The method consists in pre-staining the serum lipoproteins with a saturated solution of acetylated Sudan Black B in propylene glycol. The dye solution is added to the serum in the ratio of 1 volume of dye to 10 volumes of serum. The serum-dye mixture is allowed to stand at 25°C for 45 minutes, centrifuged, and the supernatant used for the electrophoretic separation.

2. The best conditions for the paper electrophoretic separation of the pre-stained serum were:
   Filter paper: Macherey and Nagel Nr. 2214 ff
   Buffer: veronal/Na-veronal, pH 8.6, ionic strength of 0.05
   Volume applied to paper: 30 micro-liters
   Potential gradient: 8 volt/cm
   Current: 1.5 mA/strip
   Temperature: 25°C ± 2°C
   Duration of the run: 2 hours.

3. After the electrophoretic separation the strips are dried in

the dark at 50°C and scanned at 595 mμ. The curves are analyzed by planimetry or by electronic integrators and the results expressed as percentages of the total area or total integration units. The alpha/beta ratio is calculated from these values.

4. The color in the strips is stable for 48 hours if the strips are kept in the dark. The method was shown to be very reproducible and the error in the determination of individual fractions is of the order of 3%.

5. The described method was used to determine serum lipoprotein levels of rats intoxicated with carbon tetrachloride. The animals were injected subcutaneously with 0.1 ml CCl_4 per 100 g body weight. A control group was injected with the same volume of isotonic saline.

6. Normal controls were starved for 24 hours after the injection of saline. The serum lipoproteins were determined and showed the following mean percent values for 12 animals (mean ± standard deviation):

alpha-lipoproteins: 54.6 ± 2.7

beta-lipoproteins: 45.4 ± 2.7

Beta/alpha ratio: 0.84 ± 0.09

7. The animals injected with CCl_4 were divided into 5 groups. Blood samples were collected at intervals of 24, 48, 72, 96 and 120 hours after the injection. All animals were starved for 24 hours before bleeding.

8. The animals bled 24 hours after the administration of CCl_4 (8 animals) showed the following values
alpha-lipoproteins: 28.0 $\pm$ 4.1
beta-lipoproteins: 72.0 $\pm$ 4.1
beta/alpha ratio: 2.64 $\pm$ 0.60

9. The animals bled 48, 72 and 96 hours after the administration of CCl_4 showed somewhat lower values for the beta-lipoproteins and for the beta/alpha ratio. However, these values did not differ statistically from those obtained 24 hours after the injection.

10. The animals bled 120 hours after the administration of the drug (9 animals) showed the following values:
alpha-lipoproteins: 40.9 $\pm$ 4.0
beta-lipoproteins: 59.1 $\pm$ 4.0
beta/alpha ratio: 1.46 $\pm$ 0.23
These results are statistically different from those obtained 24 hours after the injection of CCl_4. However, the beta-lipoprotein values are still above those of normal controls.

11. These results show that there is an increased concentration of beta-lipoproteins in serum of rats poisoned with CCl_4. This increase stays at high levels for 96 hours and then starts falling to normal control range. Nevertheless, the levels are still above the controls 120 hours after the injection.

12. The significance of these changes are discussed and it is inferred from the work of others that CCl_4 possibly poisons the secretory mechanism by which the liver secretes triglycerides and cholesterol into the blood stream.
The increase in beta-lipoproteins in this case is explained as being due to an increased synthesis of these complexes as an alternative to elimination of excess fat accumulated in the liver, in the early stages of CCl_4 poisoning.

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APPROVAL SHEET

The thesis submitted by Luiz P. Ribeiro has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Date

Signature of Adviser